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Site-specific small molecule labeling of an internal loop in JC polyomavirus pentamers using the π-clamp-mediated cysteine conjugation

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Abstract: The major capsid protein VP1 of JC Polyomavirus assembles into pentamers that serve as a model for studying viral entry of this potentially severe human pathogen. Previously, labeling of viral proteins utilized large fusion proteins or non-specific amine- or cysteine-functionalization with fluorescent dyes. Imaging of these sterically hindered fusion proteins or heterogeneously labeled virions limits reproducibility and could prevent the detection of subtle trafficking phenomena. Here we advance the π-clamp-mediated cysteine conjugation for site-selective fluorescent labeling of VP1-pentamers. We demonstrate a one-step synthesis of a probe consisting of a bio-orthogonal click chemistry handle bridged to a perfluoro-biphenyl π-clamp reactive electrophile by a polyethylene glycol linker. We expand the scope of the π-clamp conjugation by demonstrating selective labeling of an internal, surface exposed loop in VP1. Thus, the π-clamp conjugation offers a general method to selectively bioconjugate tags-of-interest to viral proteins without impeding their ability to bind and enter cells.

JC Polyomavirus (JCPyV) is the causative agent of progressive multifocal leukoencephalopathy (PML), a severe central nervous system disease that appears in immunocompromised individuals and is a known side effect of several approved biologic drugs.[1-3] The majority of the population becomes infected with JCPyV by adulthood. Despite chronic infection of kidney tissue, healthy individuals remain asymptomatic throughout the course of their lives.[4] In some immunosuppressed individuals, however, viral spread to the central nervous system results in destruction of astrocytes and oligodendrocytes, ultimately leading to fatal neurodegeneration, typically within 1-2 years.[5,6] The development of antiviral therapies for JCPyV-associated neurodegeneration has been hampered by incomplete understanding of viral pathogenesis, including entry and transport within cells.[7]

JCPyV is a nonenveloped virus comprised of 360 copies of the major capsid protein VP1, which assemble with pentameric symmetry into 72 capsomers in a T=7d configuration.[8] Each of these capsomers, or pentamers, are linked to neighbouring pentamers through elaborate C-terminal extensions and disulfide bonds. As is typical among viral capsid proteins, the standard conformation of VP1 is that of an antiparallel β-jellyroll fold. Expression of a truncated version of VP1, lacking residues on the N- and C-termini results in the formation of isolated viral pentamers, which retain the same overall capsomere conformation of the intact virus and are a useful model system for exploring the structure and function of polyomaviruses.[8]

Mechanistic details of JCPyV's cellular entry and trafficking have been explored using a truncated VP1-pentamer and live-cell fluorescence microscopy.[9] Two strategies have commonly been employed to label viral capsids: (i) genetic encoding of a fluorescent protein (FP) in frame with a structural protein within the viral capsid, and (ii) chemical labeling of purified virions with small molecule fluorophores.[10-12] While genetically encoded FPs have been informative for studying large viruses such as herpes virus, the

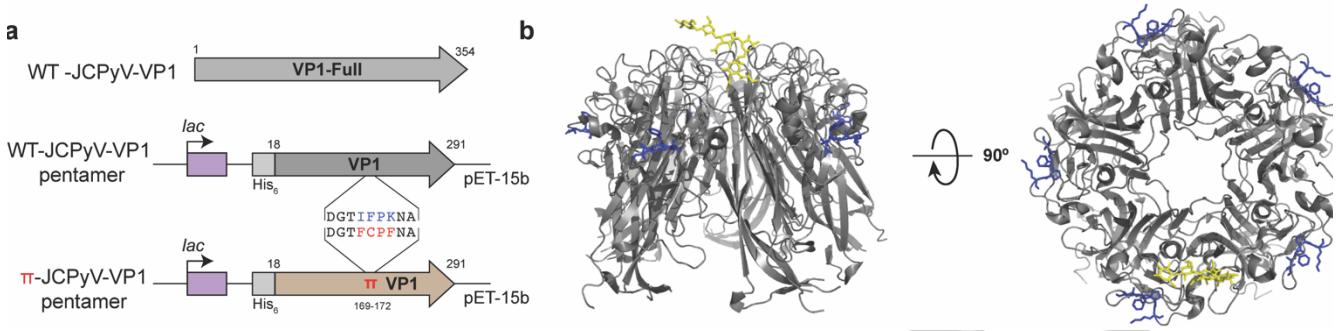


Figure 1. Design of constructs used for encoding the π-clamp sequence in JCPyV-VP1. (**a**) Schematic representation of the JCPyV VP1 with (top) WT JCPyV full length VP1 protein, (middle) WT-JCPyV-VP1P with N- and C-terminal deletions and results in formation of pentamers, and (bottom) π-JCPyV-VP1P with the π-clamp sequence encoded on a surface exposed loop (**b**) Cartoon representations of the crystal structure of JCPyV-VP1p. The backbone of five monomers assembled into a pentamer are shown in grey, the target site for π-clamp sequence insertion shown in blue, the receptor binding motif LSTc is shown in yellow.

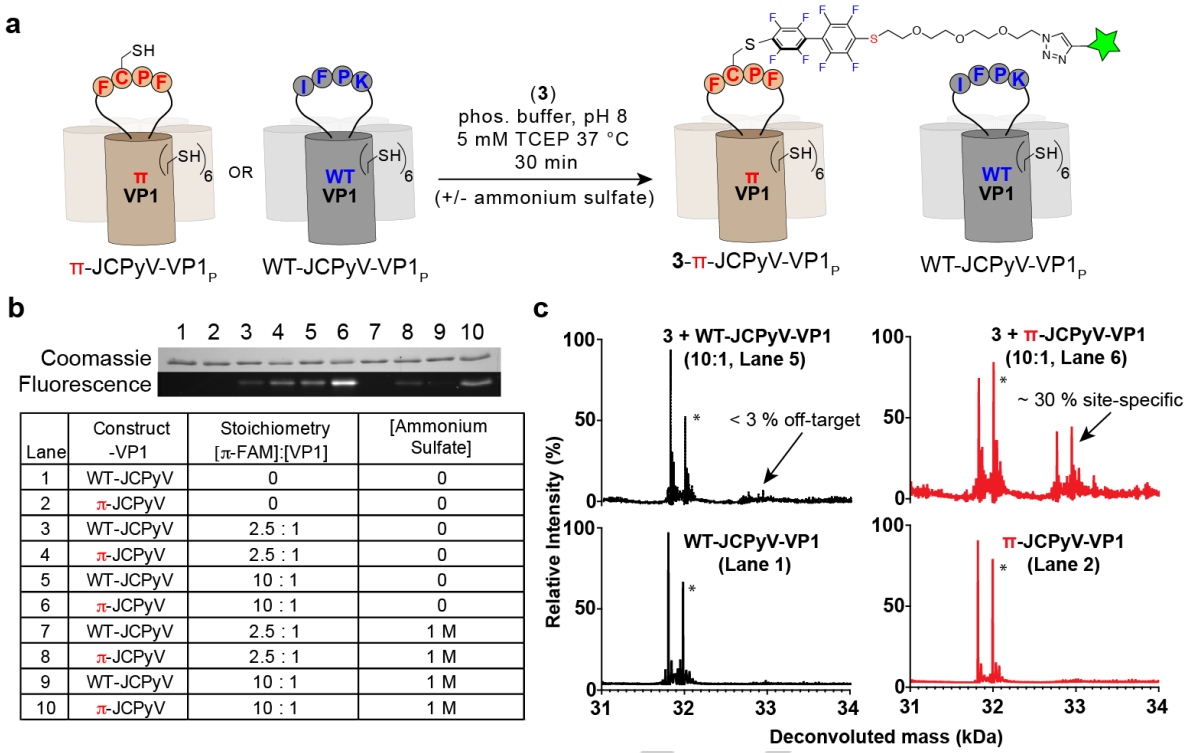
relatively large size of the FP is limiting for a small virus such as JCPyV, as viral protein function and interactions with host cellular factors are likely perturbed by steric hindrances.[13,14] The use of small molecule fluorophores addresses the size complications of fusion FPs, but unfortunately is plagued by limited selectivity that result in modification of functionally critical amino acid residues and/or heterogeneously labeled virions. The cysteine residues of JCPyV in principle offer a location for the introduction of small molecule fluorophores, however, they are known to be critical for the biological function of the virus.[9,15] JCPyV also has 11 surface exposed lysine residues per VP1, thus, complete labeling would result in 3,960 fluorophores per virion, likely resulting in loss of fluorescence through self-quenching and potentially destroying important structural or functional roles of a number of lysine residues. To counteract this, previous approaches have used sub-stoichiometric labeling, which prevents self-quenching but leads to a large degree of batch-to-batch variability and an heterogenous sample with a variable number of labels per virion.[9,16]

Herein, we demonstrate a method for fluorescent labeling of VP1-pentamers that is both minimally perturbing to the VP1 amino acid sequence and results in defined homogenous batches of virions for improved live-cell microscopy studies of JCPyV infection. Towards this goal, we advance the π-clamp-mediated cysteine conjugation for selective labeling of VP1-pentamers. Discovered by Pentelute and coworkers, the π-clamp amino acid sequence Phe-Cys-Pro-Phe catalyzes selective, orthogonal modification of the embedded cysteine residue with a perfluoro-biphenyl electrophile.[17] Previous demonstrations of the π-clamp conjugation have been restricted to conformationally flexible short peptides (<20 amino acids) and N- or C- terminal regions of proteins, with an emphasis on the synthesis of antibody conjugates.[17,18] Furthermore, perfluoronated biphenyl probes required to modify the π-clamp cysteine are not commercially available. We demonstrate a concise synthesis of an easily tunable π-clamp electrophile (**1**, **Scheme 1**) that can be modified with virtually any fluorescent moiety or other tag of interest using click chemistry. We genetically encoded the π-clamp sequence into a non-conserved loop region of the VP1-pentamer and showed site-specific labeling with our newly synthesized probe. Previously developed reaction conditions were optimized for selectively incorporating on average a single fluorophore per VP1-pentamer, enabling fluorescent microscopy imaging of viral entry and trafficking in human fetal glial cells.

Design of a π-clamp containing JCPyV VP1 pentamer (π-JCPyV-VP1$_P$). In order to demonstrate the feasibility of using the π-clamp conjugation to site-specifically install fluorescent labels on polyomavirus proteins, we generated recombinant VP1 pentamers containing the π-clamp sequence (F-C-P-F). These pentamers were designed using a truncated version of VP1 that is unable to assemble into virion-like particles and can be expressed in bacteria **(Figure 1a)**.[8] VP1-pentamers have been shown to bind to similar receptors as intact virions and recapitulate early steps in viral entry.[8,9]

JCPyV VP1 protein adopts a β-jellyroll structure with the beta strands buried in the center of the protein and the surface of the virus composed of long loops connecting the strands (**Figure 1b**).[8] As the N- and C-termini of JCPyV VP1 and other polyomaviruses are not accessible in the fully assembled virus, a surface-exposed loop was targeted for π-clamp labeling. An amino acid alignment of JCPyV VP1 with known polyomavirus VP1s shows low sequence identity in this EF loop region, further suggesting that this loop may not have conserved function (**Figure S1**). Previous work in the related mouse polyomavirus (MPyV) demonstrated that the EF loop is surface-exposed and was able to accommodate small peptide inserts.[19] Alignment of JCPyV VP1 to the MPyV structure shows that this loop is also surface exposed, making it an ideal site for mutagenesis (**Figure S2**). Finally, the EF loop appears to be far from the receptor binding pockets, suggesting that insertion of the π-clamp sequence and subsequent conjugation of a label may not impact binding or cellular entry of recombinant VP1 pentamers.[8]

Examination of the amino acid sequence of the EF loop revealed the presence of a proline residue in position 171 (**Figure 1a**), thus, the introduction of the π-clamp sequence into the EF loop would only require three mutations: I169F, F170C and K172F. With the exception of K172F, these are conservative mutations (**Figure 1a**). The resulting construct was termed π-JCPyV-VP1$_P$. Comparing the x-ray crystal structure of wild-type JCPyV VP1 to a predicted homology model of π-JCPyV-VP1$_P$ suggested that the overall changes to the tertiary structure of VP1 are small (**Figure S3**).



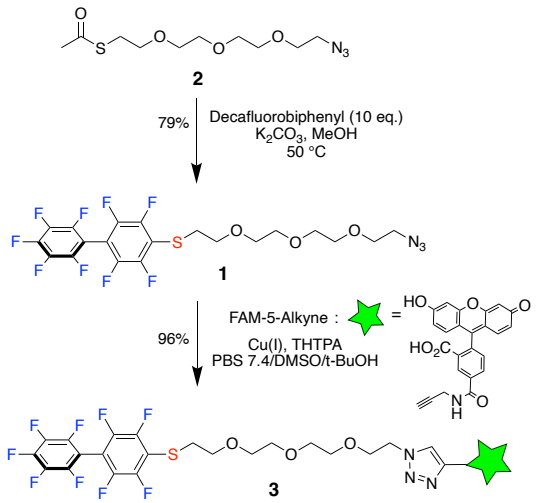
Lane	Construct -VP1	Stoichiometry [π-FAM]:[VP1]	[Ammonium Sulfate]
1	WT-JCPyV	0	0
2	π-JCPyV	0	0
3	WT-JCPyV	2.5 : 1	0
4	π-JCPyV	2.5 : 1	0
5	WT-JCPyV	10 : 1	0
6	π-JCPyV	10 : 1	0
7	WT-JCPyV	2.5 : 1	1 M
8	π-JCPyV	2.5 : 1	1 M
9	WT-JCPyV	10 : 1	1 M
10	π-JCPyV	10 : 1	1 M

Figure 2. Optimization of π-clamp reaction conditions. (**a**) Schematic of general reaction conditions for **3** labeling of JCPyV. (**b**) Analysis of π-clamp reactions by SDS-PAGE with in-gel fluorescence. (**c**) Deconvoluted intact protein mass spectrometry of labeling reactions. *Second species resulting from incomplete N-terminal processing for removal of methionine in recombinant expression.

Design and synthesis of a π-clamp reactive probe. In the original development of the π-clamp conjugation methodology, the Pentelute group used dual-modified peptides, where the perfluoro-biphenyl electrophile was installed on a central cysteine residue in the peptide, and a fluorescent dye or affinity tag was installed at the N-terminus. The π-clamp conjugation is driven by the sidechains of each phenylalanine flanking the cysteine in Phe-Cys-Pro-Phe, which are kinked inward by the proline, forming a catalytic clamp for the incoming perfluoro-biphenyl moiety. Although the peptide component of the original probes aided in the aqueous solubility of the nonpolar perfluoro-biphenyl moiety, its preparation required several steps of small-scale solid phase peptide synthesis.[17,20] The Williams and Pentelute groups have synthesized π-clamp reactive probes consisting of a polyethylene glycol (PEG) linker connecting either a TAMRA dye or biotin to the perfluoro-biphenyl electrophile, demonstrating the potential of non-peptide, PEG-based π-clamp probes to selectively label protein termini.[21, 22] The Williams group also showed that dual fluorescent modifications were possible by combining the π-clamp labeling system with Sortase-mediated chemical tagging. However, demonstrations of the π-clamp conjugation on non-terminal regions of full proteins have not been reported and will be important for potential applications where the terminal regions are not exposed to solvent or are otherwise critical for function.

Here we synthesize a new π-clamp probe (**1**, **Scheme 1**) that enables facile exchange and optimization of the tag functionality opposite of the π-clamp reactive perfluoro-biphenyl via click chemistry. We envisioned that a triethyleneglycol linker would effectively bridge the click chemistry reactive azide to the electrophilic perfluoro-biphenyl while providing necessary enhancement of the probe's water solubility. Compound **1** was prepared in one step and 78 % yield by reacting thioacetate **2** with decafluorobiphenyl and potassium carbonate in methanol (**Scheme 1**). Thioacetate **2** is commercially available or easily prepared from tetraethylene glycol in four steps and 39% overall yield (see, Supporting Information). In situ deprotection of the thiol group in degassed methanol and the use of a large excess of decafluorobiphenyl greatly reduced the formation of disulfide biproduct. To demonstrate the utility of compound **1** to serve as a precursor to a variety of π-clamp reactive perfluoroaromatic probes, we reacted it with 5-FAM alkyne using copper (I) catalyzed click chemistry to give **3** in 96% yield.

Scheme 1. Synthesis of π-clamp reactive probes **1** and **3**.



Optimization of π-clamp conjugation reaction conditions. Previous demonstrations of the π-clamp conjugation were restricted to unstructured N- and C- terminal regions of the proteins of interest. To optimize reaction conditions for the internal loop region of π-JCPyV-VP1, we investigated reaction time, ratio of π-JCPyV-VP1 (monomer) to **3**, and addition of the chaotrope ammonium sulfate, which was shown by Pentelute and coworkers to promote selectivity in certain π-clamp conjugations (**Figure 2a**).[18] The extent of labeling was determined by SDS-PAGE and intact protein mass spectrometry (**Figure 2b-c** and see Supporting Methods). Initially, we examined reaction duration to find the optimal time that resulted in selective modification of the π-JCPyV-VP1P π-clamp cysteine over native cysteines in JCPyV-VP1P. This analysis demonstrated that after 30 minutes, roughly 30 % of π-JCPyV-VP1 was modified with **3**, whereas the control, WT-JCPyV-VP1P (lacking the π-clamp sequence) showed maximally 3 % labeling (**Figure 2**). JCPyV-VP1 contains six cysteines: two of which, C41 and C96, are solvent exposed in the crystal structure. It is likely that the labeling seen in WT-JCPyV-VP1P is due to off-target modification of one of these cysteines. Next, the relative ratios of protein to **3** and the addition of ammonium sulfate were investigated. These experiments demonstrated that a **3** to π-JCPyV-VP1 ratio of 10:1 without ammonium sulfate produced the optimal degree of labeling and selectivity to afford one fluorescent label per VP1 pentamer on average (**Figure 2b-c**).

Cell binding and uptake of 3-π-JCPyV-VP1 pentamers. We next sought to verify that neither inclusion of the π-clamp sequence nor conjugation to the probe impact binding or uptake of VP1 pentamers in a human fetal glial cell line (SVGA) that is susceptible to viral infection.[23] Inoculation media or either wildtype (negative control) or π-clamp containing VP1 that had previously been reacted with probe were added to cells that had been chilled to prevent endocytosis.

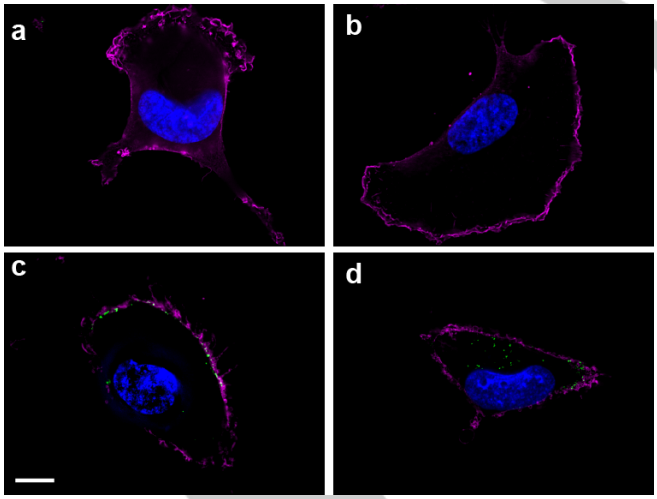


Figure 3. Fluorescence imagining of **3**-π-JCPyV-VP1P. Susceptible SVG-A cells were chilled to prevent endocytosis and inoculated with tissue culture media containing (**a**) No VP1, (**b**) WT-JCPyV-VP1P (**c**) **3**-π-JCPyV-VP1P. Cells were fixed at 0 h (**b**) and (**c**), or warmed to allow endocytosis for 1 h and then fixed (**d**). Texas-red wheat germ agglutinin was used to stain the plasma membrane (magenta) and Hoechst was used to stain the nucleus (blue) and **3**-π-JCPyV-VP1P is shown (green). Scale bar represents 10 μm.

Cells were either fixed and imaged to show binding to the cell membrane, or allowed to warm to 37 ºC for 1 h to allow for endocytosis. Following fixation, cells were incubated with Texas-red labeled wheat germ agglutinin to label the plasma membrane (**Figure 3**). This analysis demonstrates that **3**-π-JCPyV-VP1P are able to bind and enter cells in a similar manner to previously published reports (**Figure 3**).[9,16]

Here we show that the π-clamp conjugation can be used to selectively modify JCPyV VP1 to enable fluorescence microscopy applications. We present a succinct synthetic strategy for access to small-molecule π-clamp probes, where a modular approach enables facile adjustment of probe properties (i.e. solubility, length, fluorescent tag). Our results also demonstrate, to the best of our knowledge for the first time, that the π-clamp conjugation is effective for labeling not only terminal locations, but also internal sites on large proteins. Lastly, the π-clamp offers an effective means to bioconjugate probes to viral proteins while retaining the ability to bind and enter cells. Methods to specifically label and therefore distinguish subunits within an assembly offer the potential to study subunit dependent phenomena, which has not been possible with previously used indiscriminate dye labelling techniques (using activated NHS-ester or maleimide conjugation chemistry).

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Keywords: bioorthogonal • π-clamp • click chemistry • fluorescent probes • protein modifications • viruses

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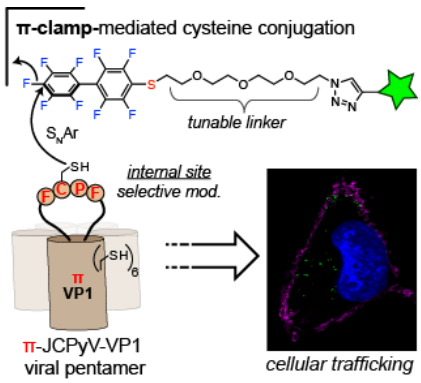

This communication advances the recently developed π-clamp-(Phe-Cys-Pro-Phe)-mediated cysteine conjugation for fluorescent labeling of JC Polyomavirus (JCPyV) pentamers. We show a concise (two steps) synthesis of a small molecule π-clamp reactive probe that is easily tunable for a broad set of fluorescent reporters, solubility, and fluorophore distance from the modification site. With this probe we demonstrate labeling in a structured loop region of a protein and visualize cellular entry of JCPyV viral particles.

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